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iPS cells for transplantation

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## Abstract

### Purpose of review

The induced pluripotent stem (iPS) cells from patient's somatic cells could be a useful source for drug discovery and cell transplantation therapies. However, there are still several problems to be solved in terms of safety concerns. We herein summarize the current knowledge about iPS cells and the obstacles that must be overcome before the cells can be used for medical applications.

### Recent findings

Recent progress has enabled us to generate integration-free iPS cells from non-invasive tissues. However, several studies have also uncovered differences in iPS cells and ES cells in terms of gene expression, epigenetic modification, and differentiation potentials. Tissue origin affects the quality of iPS cells. However, in a rodent disease model, the transplantation of differentiated cells derived from iPS cells ameliorated their symptoms. Methods for gene correction and direct cell fate switching have also been reported. The successful generation of HLA-typed iPS cells has been described. These findings should therefore facilitate the further application of iPS cells.

## Summary

Human iPS cells are able to provide functional neuronal cells, blood cells, and retinal cells, which would be a useful source for transplantation. Although recent reports have shown that it may be possible to overcome several difficulties associated with iPS cells, further improvements are needed, not only regarding safety aspects, but also in quality, before they can be medically applied.

## Introduction

Reprogramming of somatic cells into pluripotent stem cells has been reported by introducing a combination of several transcription factors (Oct3/4, Sox2, Klf4, and c-Myc)(1). The induced pluripotent stem (iPS) cells from patient's somatic cells could provide a useful source of cells for drug discovery and cell transplantation therapies. However, most human iPS cells have been made using viral vectors, such as retroviruses and lentiviruses, which integrate the reprogramming factors into the host genomes and may increase the risk of tumor formation (2). To overcome the safety concerns about iPS cell generation, several non-integration methods have been reported, such as transient expression of the reprogramming factors using adenoviral vectors or plasmids, and direct delivery of reprogramming proteins and RNAs (3-6). These approaches could decrease the risk of genomic alteration, however, recent studies raised other obstacles in the way of future medical applications.

Human iPS cells are able to generate neuronal cells, blood cells, and retinal cells (7-9). However, there have been some reports that human iPS cells show attenuated differentiation potential into neuronal or hematopoietic lineages in comparison to ES cells (10, 11). These studies indicate the limited applications of human iPS cells and suggest the need for improvement of reprogramming, not only in

terms of safety, but also in quality. We herein summarize the current knowledge and problems associated with the development of iPS cells for medical applications.

## **Development of an efficient non-integrated method and derivation of iPS cells from non-invasive tissues**

The first iPS cells were established by delivery of transgenes, Oct3/4, Sox2, Klf4, and c-Myc, by MMLV (Moloney Murine Leukemia Virus)-based retroviral vectors (1).

Retroviruses can robustly infect fibroblasts and introduce their RNA genome into the host genome by reverse transcriptase. The resulting iPS cells had several genomic integrations of the transgenes. This enables effective reprogramming of somatic cells, however, the integrated sequences alter the genomic organization of iPS cells and can induce some abnormalities (12). One of the reprogramming factors, c-Myc, is a well-known proto-oncogene, and its reactivation could give rise to transgene derived-tumor formation (2). Because of these concerns, several improvements in the transduction methods have been studied for making non-integrated iPS cells. Generation of non-integrated iPS cells was first reported in mice using an adenoviral vector and by using a plasmid vector (3, 4), and then it was demonstrated that it was possible using human cells and viral vectors (adenovirus), DNA vectors (PiggyBac vector, episomal

plasmid, and minicircle vector), and direct protein delivery (13-18). Although these methods can avoid genomic alterations, most of them are highly inefficient and largely impractical. Recent progress was made by the employment of the non-transmissible Sendai virus vector for reprogramming (19). The Sendai virus is a minus-strand RNA virus and does not have the risk of integrating into the host genome. Fusaki et al. mixed 4 different Sendai viral vectors encoding different reprogramming factors, and transfected them into human fibroblasts. The viral vectors could constitutively replicate their RNA genome in the cytoplasm of the infected cells, to give a high reprogramming efficiency of up to 1%. The established iPS cells still contained viral RNA, however, the RNA were diluted and disappeared during cell growth. This elimination can be enhanced by using temperature-sensitive mutants and by antibody-mediated negative selection utilizing viral antigens expressed on the cell surface.

Direct delivery of synthetic mRNA encoding reprogramming factors also induced iPS cell generation at high efficiency (6). To suppress the activation of the cellular antiviral response, mRNAs were synthesized with modified residues of 5-methylcytidine triphosphate and pseudouridine triphosphate. In addition, a 5' guanine cap was incorporated by inclusion of a synthetic cap analog to increase the RNA half-life in the cytoplasm. After purification, the RNAs were mixed and transfected into

human fibroblasts. This method provided rapid and large-scale generation of iPS cells. The estimated efficiency was beyond 4% when using a 5 factor cocktail (Oct3/4, Sox2, Klf4, c-Myc, and LIN28) and low oxygen conditions. The efficiency greatly surpassed that of all other reported protocols. The RNA-driven iPS cells showed pluripotency in their differentiation into cell types of all three germ layers. The method can also be applied to facilitate cell differentiation. Synthetic RNA encoding a myogenic factor, MYOD, enhanced the terminal differentiation of iPS cells into myotubes.

Many additional reprogramming factors have been reported that seem to affect both the induction efficiency and characteristics of iPS cells. For example, Hanna and colleagues found that some reprogramming factors, such as Lin28 and shRNA for p53, mainly regulate the reprogramming efficiency through the control of cell proliferation (20). In contrast, Nanog seems to enhance the efficiency of reprogramming by affecting the reprogramming process itself. Tbx3 was reported to improve the quality of mouse iPS cells, as it facilitated the germline transmission efficiency (21). Modification of cell signaling (by Wnt, inhibition of TGFbeta, and inhibition of MEK), or treatment with chemicals/natural compounds (valproic acid, 5'-azacytidine, thiazovivin, and vitamin C) have also been employed to develop better iPS cells (22-26). One company in the US has applied for a patent based on a process that generates iPS cells by using small



molecules. Although more detailed analyses are needed, one of these approaches could potentially be ideal for the future generation of iPS cells.

The origin of the iPS cells seems to be important, especially for humans. It is necessary to consider clinically available sources with respect to minimizing the invasiveness of collection and making them more easily accessible. Initially, iPS cells were established from fibroblasts (27, 28). Thereafter, numerous tissues sources have been investigated, and iPS cells can now be established from various tissues including keratinocytes, mesenchymal cells in fat, oral mucosa, molar teeth, peripheral blood, and cord blood (29-34). The generation of iPS cells from hematopoietic cells is noteworthy, because there are many established blood banks which already determine the HLA types of their blood. Seki et al. reported efficient generation of iPS cells from peripheral blood using Sendai viral vectors (35).

### **Genetic correction**

iPS cells established from individual patients can provide a promising cell source for cell therapy, as they do not elicit immune rejection. However, when the patient suffers from a genetic disease, the cells need genetic correction. In general, human pluripotent stem cells are more difficult to modify compared to mouse ES cells. However, recent

studies have demonstrated effective gene modification of human pluripotent cells. Hockemeyer et al. employed a zinc-finger nuclease (ZFN) for genome editing (36). They designed a ZFN that recognized adjacent genomic sequences of the correction locus, by fusing the FokI nuclease domain on a DNA recognition domain composed of engineered C2H2 zinc-finger motifs. After transduction into iPS cells, the chimeric proteins bind at flanking genomic regions, dimerize with the nuclease domains, and cut the genomic DNA. Then a donor DNA, which is homologous to the target, is incorporated by homology-directed repair. This approach has several limitations, however, it can supply a powerful tool for gene correction.

Another method for efficient genetic correction is to use human iPS cells which show mouse ES cell-like characteristics. Conventional mouse and human ES cells share many characteristics, such as unlimited proliferation and differentiation potential into cells of all three germ layers, however, the cells have a significant number of distinct features including morphology, gene expression pattern, and cytokine dependence. Therefore, mouse and human pluripotent cells are termed “naïve” and “primed” pluripotent cells, respectively. Naïve mouse ES cells form compacted dome-shaped colonies in culture. They are largely dependent on external LIF signaling and have a high proliferation rate. On the other hand, human-type primed ES cells grow as flat

colonies, and are predominantly dependent on bFGF signaling, and have slow growth. Although both types of stem cells can differentiate into cell types from all three germ layers, the mouse naïve stem cells seem to have higher pluripotency, which enables them to contribute to chimera formation and germ cell lineage. Recently, the generation of human naïve ES and iPS cells has been reported in the presence of LIF and chemical supplementation (37, 38). In contrast to primed ES cells, the human naïve ES cells showed similar properties as mouse naïve ES cells with regard to their morphology, gene expression profile, and signaling pathway dependence. Interestingly, the human naïve ES cells also allowed efficient gene targeting by conventional homologous recombination methods, similar to the mouse naïve ES cells, which could provide a powerful method for gene repair. However, the culture conditions for naïve human pluripotent cells still needs to be optimized, as they easily revert to their primed state. Further studies will be necessary to enable stable handling of naïve human ES cells.

### **HLA banking**

It is estimated that it would take at least a few years to establish clinical grade cells from a patient's own somatic cells. Each of the derived cell lines should be assessed for their medical stability, safety and efficacy. Thus, while ES cells might represent a novel

treatment approach for currently incurable diseases, it is necessary to consider the applications of iPS cells from an economical view point. Complete tailor-made iPS cell therapy would cost too much for most people. From this point of view, a banking system for iPS cells is proposed. To decrease immune rejection, iPS with various HLA haplotypes should be collected, similar to the way whole blood is currently banked.

Based on the experiences with organ transplantation, it appears that the most important HLA molecules to match are the HLA class I molecules, HLA-A and HLA-B, and the class II molecule, HLA-DR. HLA matching of these loci can reduce the incidence of acute rejection, and has improved transplant survival. Estimations of the necessary stem cell bank size have been calculated for the Japanese and the UK populations (39, 40). If 170 lines of iPS cells were randomly established, 80% of such patients would be able to find a donor line with a single mismatch at one of the three HLA loci, HLA-A, -B, and -DR, with a better match found within the Japanese population. A bank size of 150 lines could therefore provide an acceptable match for 84.9% of the UK population.

Importantly, when iPS cells are established from HLA homozygous cells, a smaller bank size of only 50 lines is expected to provide a three-locus match for 90.7% of the Japanese population (41). To identify at least one homozygote for each of the 50 different HLA haplotypes, it would be necessary to examine an HLA-type database of

24,000 individuals. This would be possible if the iPS bank cooperated with other banks, like cord blood banks and bone marrow banks. Generation of iPS cells from HLA-typed cells has already reported, thus supporting the feasibility of such a project. Tamaoki et al. focused on dental pulp cells, as they are easily obtained from extracted teeth, and such cells are generally considered to be medical waste, and can be expanded under simple culture conditions (42). Determination of their HLA types revealed that 2 out of 107 lines of dental pulp cells were homozygous for HLA-A, B, and DR loci.

### **Epigenetic memory of the source tissue**

The origin of iPS cells affects their characteristics, including their safety and their differentiation efficiency. Miura et al. differentiated several mouse iPS cell lines into neural precursor cells in vitro and evaluated their safety by transplantation into immunodeficient mice (43). The iPS cells they used were derived from various tissues, such as embryonic fibroblasts, adult tail fibroblasts, liver, and stomach. Although iPS cells from embryonic fibroblasts and liver tissue efficiently differentiated into neural lineage cells, iPS cells from tail fibroblasts remained significantly undifferentiated after being treated using the same differentiation protocol, which resulted in teratoma formation after transplantation. Interestingly, the tumorigenicity was largely associated

with their origin, but not with inclusion of the c-Myc transgene. When a less tumorigenic iPS cell line was selected, the differentiated neural precursor cells could ameliorate the symptoms of spinal cord injury in a mouse model (44). Kim et al. also reported the effects of cell origin on the differentiation potential of iPS cells (45). In their study, iPS cells that originated from blood cells could easily differentiate into the hematopoietic lineage, whereas iPS cells from a neuronal or fibroblast lineage showed limited differentiation potential. As treatment with inhibitors of DNA methyltransferase (5'-azacytidine) and histone deacetylase (trichostatin A) improved the hematopoietic differentiation capacity of iPS cells of neural origin, the epigenetic status appears to be important for the memory of iPS cells. In fact, several differentially methylated DNA regions (DMR) have been reported (46-48). Studies of the relationship between DMR and differentiation potential are therefore needed to determine the characteristics of iPS cells before inducing their differentiation. Some scientists believe that human naïve ES/iPS cells have less diversity than primed cells, as they are much closer to the actual pluripotent state and would therefore acquire full reprogramming more easily.

### **Direct conversion**

The generation of iPS cells has clearly shown that forced expression of a few master

regulators can modify cell fate. With this in mind, several studies have been reported showing the direct conversion from one somatic cell type into another cell type without the use of stem cells. The generation of neuron-like cells from mouse fibroblasts was accomplished by transduction of several transcription factors including *Ascl1*, *Brn2*, and *Myt1l* (49). The induced neuronal cells expressed multiple neuron-specific proteins, generated action potentials, and formed functional synapses. Ieda et al. demonstrated derivation of cardiomyocyte-like cells from cardiac or dermal fibroblasts by transduction of three developmental transcription factors, *Gata4*, *Mef2c*, and *Tbx5* (50). The cells termed “induced cardiomyocyte-like cells” expressed cardiac-specific markers, had a global gene expression profile similar to cardiomyocytes, and contracted spontaneously. The authors also transplanted fibroblasts into immunosuppressed mouse hearts after gene transduction and noted the formation of sarcomeric structures within 2 weeks. This approach represents one possible way to achieve transplantation. On the other hand, Zhou et al. reported direct *in vivo* induction of pancreatic exocrine cells to beta cells by transduction of three transcription factors, *Ngn3*, *Pdx1*, and *Mafa* (51). Adenoviral vectors encoding these genes were used for delivery to the pancreas of immunodeficient mice. The converted cells were capable of secreting insulin, and ameliorated hyperglycemia in streptozotocin-treated diabetic mice. As was observed for

iPS cells, these converted cells would have some epigenetic memory of their origin. Further studies are required, especially investigations regarding safety, before these approaches can be concluded to provide an alternative method for generating specific differentiated cells for use in medical treatment.

## Conclusion

Artificial stem cells, iPS cells, are established by the modification of the intracellular and extracellular environments. The iPS cells seem to have pluripotency, as mouse and rat iPS cells can produce chimeric animals. A tetraploid complementation experiment revealed that mouse iPS cells have the ability to develop into a full-term mouse autonomously (52, 53). These data indicate that differentiated cells derived from iPS cells can at least partially replace the biological functions of various cells types. Several therapeutic attempts have been reported in rodent models, such as treatment of humanized sickle cell anemia, Parkinson's disease, and spinal cord injury (44, 54, 55). Although many problems still remain to be resolved, iPS cells may be applicable for medical treatment in the relatively near future. Studies of disease pathogenesis and drug discovery have already been launched, and the results thereof could provide important relief to countless people throughout the world who are currently suffering from



incurable diseases or injuries.

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